



Alternation of Pituitary-Testicular Axis in the Nile Tilapia (*Oreochromis niloticus*) Exposed to Dimethoate: An Immunohistochemical and Hormonal Study

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ABSTRACT

The adverse effects of organophosphate dimethoate on testis morphology, luteinizing hormone (LH) cells structure and hormonal assay of the Nile tilapia (*Oreochromis niloticus*) were examined. Adult male tilapia, average body weight 150.0 ± 23.3 g, were exposed to environmentally relevant (0.03 mg/L⁻¹) and sublethal (5.00 mg/L⁻¹) doses of dimethoate for 15 and 30 days. Fish exposed to 0.03 mg/L⁻¹ dimethoate exhibited significant decrease in the serum LH. While, 5.00 mg/L⁻¹ dimethoate revealed significant increase in serum LH. Follicle stimulating hormone (FSH) displayed significant increase in 5.00 mg/L⁻¹ group exposed for 15 days. However, a significant reduction was observed in 0.03 mg/L⁻¹ group exposed for 30 days. Histological structure of the testes of the control tilapia showed numerous spermatogenic cysts; lumina filled with spermatozoa and little interstitial tissue. Fish exposed to dimethoate for 15 days showed scarcely spermatozoa, narrow seminiferous tubules and increase in the interstitial connective tissue. After 30 days, 5.00 mg/L⁻¹ exposed fish showed progressive reduction of spermatozoa, empty tubules and a degenerative cell debris in the lumen of the seminiferous tubules. Immunohistochemically, the LH cells were localised in proximal parse distalis of the tilapia adenohypophysis. LH cells were isolated, clustered or formed cord of cells surrounding the neurohypophyseal branches. In dimethoate exposed fish, LH cells displayed a reduction in size and mass distribution in the adenohypophysis. The cytoplasm of the LH cells were vacuolated and showing the signs of degeneration. The changes appeared in testicular structures and LH cells of the *O. niloticus* were in a dose and time-dependent manner. Based on these observations, sublethal or environmentally relevant concentrations of dimethoate exhibited an endocrine disruptive action at pituitary-testicular axis of Nile tilapia that might lower its reproductive potential.

Introduction

The hazardous use of insecticides has been accentuated by the sharp rise in their use in agriculture, industry, householders, modern industrial process and governments (Sayim, 2007). It reaches the water resources from the agricultural runoff or industrial effluents, and posing hazardous effect to the aquatic biota; including both vertebrates and invertebrates. Dimethoate; an organophosphorus pesticide (OP); is massively used, replacing organochlorine due to their less persistent life and

easy detoxification in animal tissues (Quaraishi, 1977). Endocrine disrupting chemicals (EDCs) were defined as an exogenous agent that interferes with synthesis, secretion, metabolism, binding action, or elimination of hormones responsible for homeostasis, reproduction and developmental process. Dimethoate is one of the EDCs (Astiz *et al.*, 2009) exerting adverse effects on the endocrine and reproductive systems (Jobling *et al.*, 1998). Exposure of fish to EDCs alter their reproductive physiology and morphology (Kim, 1998; Tyler *et al.*, 1998).

Experimental studies have shown that OP can act as EDC because of their capacity to impair the male sexual hormone profile (Kang *et al.*, 2004).

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Additionally, Walsh *et al.* (2000) reported that, dimethoate can inhibit the steriodogenesis by disrupting the transcription of the STAR protein. In sublethal toxicity, the hypothalamo-pituitary-gonadal axis is considered a good biomarker for evaluating the adverse effects of the pollutants (Banaee *et al.*, 2008; Kavitha and Rao, 2009). It was noticed that long term exposure of male *Oreochromis niloticus* to dimethoate included food (1.6 mg/kg) altered the male sex hormones with degeneration and necrosis of the testicular tissue (Abd El-Gawad *et al.*, 2011).

Identification and distribution of luteinizing hormone (LH) cells in the pituitary have been established by immunocytochemical techniques in many teleost fish; *O. niloticus* (Kasper *et al.*, 2006); *Sparus aurata* (Ayala *et al.*, 2003) and Mediterranean yellow tail, *Seriola dumerilii* (García-Hernández *et al.*, 1996). On the other hand, detailed descriptions of the normal histological structures of teleost testis have been compiled (Hassanin *et al.*, 2002; El-Sakhawy *et al.*, 2011). The aim of the present study was to elucidate the effects of sublethal and environmentally relevant concentrations of dimethoate on hormonal assay, testis morphology and pituitary LH cells of mature Nile tilapia detected by immunohistochemical technique.

Materials and methods

Fish

Sixty adult male Nile tilapia (*Oreochromis niloticus*) with an average weight 150.0 ± 23.3 g obtained at May, 2013 from a private fish hatchery were used in this study. Fish were acclimated to the laboratory conditions for two weeks in fiberglass tanks (750 L) with continuous aerated dechlorinated static water at $26.0 \pm 2.0^\circ\text{C}$ under a 12 hours/12 hours light/dark cycle at the wet-lab of Fish Diseases and Management, Faculty of Veterinary Medicine, Benha University, Egypt. Fish were daily fed with commercial pellet diet (Joe trade Company, Egypt) at 3% of their initial body weight.

Preparation of dimethoate concentrations

The sublethal concentration of dimethoate 40% EC (Kafr El Zayat pesticides and chemicals CO., Egypt) was determined based on the static renewal

bioassay method following Sprague (1973). The LC_{50} (the concentration at which 50% of dimethoate exposed fish for 96 hours were died) was calculated following Finney's Probit analysis method (Finney, 1971). It was found that the LC_{50} was 25.00 mg/L^{-1} and consequently the sublethal concentration was calculated after Sprague (1973) to be 5.00 mg/L^{-1} . The environmentally relevant concentration (0.03 mg/L^{-1}) has been adopted in the present study mimicking the natural habitat of fish based on a previous work detected the residue of dimethoate in fish farm water (Abd El-Gawad, 2011).

Experimental design

Fish were exposed to dimethoate at 0.03 and 5.00 mg/L^{-1} for 15 and 30 days. Daily water exchange and reconstitution of toxin level was carried out. Fish were not fed on the day of sampling. Blood samples and tissue specimens were collected on day 15 and 30 post-exposure for hormonal analysis and histological study after anesthetization with Tricaine methane sulfonate (MS-222, Sigma, USA).

Serum samples and hormonal analysis

Blood samples were collected from 5 fish per group from the caudal vein using syringe fitted with a 27G needle without anticoagulant. Collected samples were centrifuged at $1400 \times g$ for 15 minutes and the separated serum was used for hormonal estimation of follicle stimulating hormone (FSH) and LH following Kandiel *et al.* (2013), using ELISA Kits for measurement serum levels of FSH (Catalog No: E0830f, EIAab®, Wuhan, China) and LH (Catalog No. CSB-E15791Fh, Cusabio Biotech Co., Ltd®, Wuhan, Hubei, China).

Histological examination

Testis and the pituitary glands were rapidly removed fixed in Bouin's fluid, dehydrated through graded ethanol solutions, cleared and then embedded in paraffin. Sections of $5\mu\text{m}$ thickness were stained with hematoxylin and eosin for histological examination. Other $5\mu\text{m}$ thickness sections of pituitary glands were mounted on poly-L-Lysine (Sigma, USA) coated slides for immunohistochemistry.

Immunohistochemical procedures

The changes of LH cells in the pituitary gland were determined by application of immunohistochemical technique using avidin-biotin peroxidase complex (ABC; Vectastain, Vector Laboratories, CA) according to Hsu *et al.* (1981). Rabbit anti-serum directed against human LH (anti-hLH) was obtained from National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, USA). Briefly, 5µm sections were dewaxed, rehydrated, and incubated in 3% hydrogen peroxide for 10 minutes at room temperature to quench endogenous peroxidase activity. The sections were rinsed in phosphate-buffered saline (PBS pH 7.4), then incubated with 0.1% BSA for 10 minutes at 37°C to reduce nonspecific reactions. They were then incubated overnight at 4°C with rabbit anti-hLH (1:5000 dilutions). They were rinsed with PBS (3×5 minutes) and incubated with anti-rabbit IgG second antibody for 30 minutes at 37°C; then rinsed in PBS (3×5 minutes). The sections were incubated with ABC for 1 hour at 37°C. Bound antibodies were visualized using diaminobenzidine tetrahydrochloride (Sigma-Aldrich, USA) solution for 10 minutes in distilled water. All incubations were performed in a humidified chamber. Sections were counter-stained in Myer's hematoxylin, dehydrated and mounted with DPX (Sigma, Munich, Germany). The specificity of the immunoreaction was confirmed by incubating the sections with normal rabbit serum instead of the specific antiserum.

Statistical analysis

Statistical analysis was performed with SPSS (ver. 16.0.2) software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test for comparison between different treatments. Results were reported as mean±S.E. and differences were considered as sig-

nificant when $P < 0.05$.

Results*Hormonal assay*

The results of hormonal assays of LH and FSH in male tilapia were tabulated in Table 1. Fish exposed to dimethoate at 0.03 mg/L⁻¹ for 15 and 30 days showed significant ($P < 0.05$) decrease in LH level. While, at 5.00 mg/L⁻¹, there was a significant increase in LH levels compared to the control. In the meantime, FSH showed a significant ($P < 0.05$) increase at 5.00 mg/L⁻¹ exposed group for 15 days; then returned to the normal level at 30 days of exposure. Fish exposed to 0.03 mg/L⁻¹ dimethoate showed a significant reduction of FSH level at 30 days.

Histological features of the testis

In the control group, the testis was composed of seminiferous tubules that were oriented at the right angles to the long axis of the testis and ended blindly at the periphery (Fig 1A). The tubules contained spermatogenic cysts at different stages of development and the lumen were filled with spermatozoa. The interstitial connective tissue was a thin layer of collagen fibers housing the Leydig cells and blood capillaries (Fig 1B).

In the dimethoate exposure tilapia, the testes of tilapia of (0.03 mg/L⁻¹) exposed group showed no pronounced histological changes after 15 days of exposure compared with the control. The testes in day 30 specimens appeared less packed with spermatozoa, narrow lumina and contained fewer spermatogenic cysts, when compared to the control (Fig 1C). The interstitium connective tissue was thick (Fig 1D). At sublethal dose (5.00 mg/L⁻¹), exposed fish showed scarce spermatozoa in the lumen of the seminiferous tubules with some empty

Table 1. Hormonal assay of dimethoate exposed male tilapia at concentrations (0.03 and 5.00 mg/L⁻¹) for 15 and 30 days.

Parameters	Exposure time		15 days (n=5)		30 days (n=5)	
	Control	0.03 mg/L ⁻¹	0.03 mg/L ⁻¹	5.00 mg/L ⁻¹	0.03 mg/L ⁻¹	5.00 mg/L ⁻¹
LH (IU/ml)	0.31±0.01c	0.12±0.01d	2.11±0.02a	0.12±0.02d	0.48±0.02b	
FSH (IU/ml)	0.36±0.02b	0.25±0.04b	0.98±0.12a	0.12±0.04c	0.34±0.05b	

n= number of fish

Values (means ±SEM) with different letters in the same row are significantly different ($P < 0.05$).

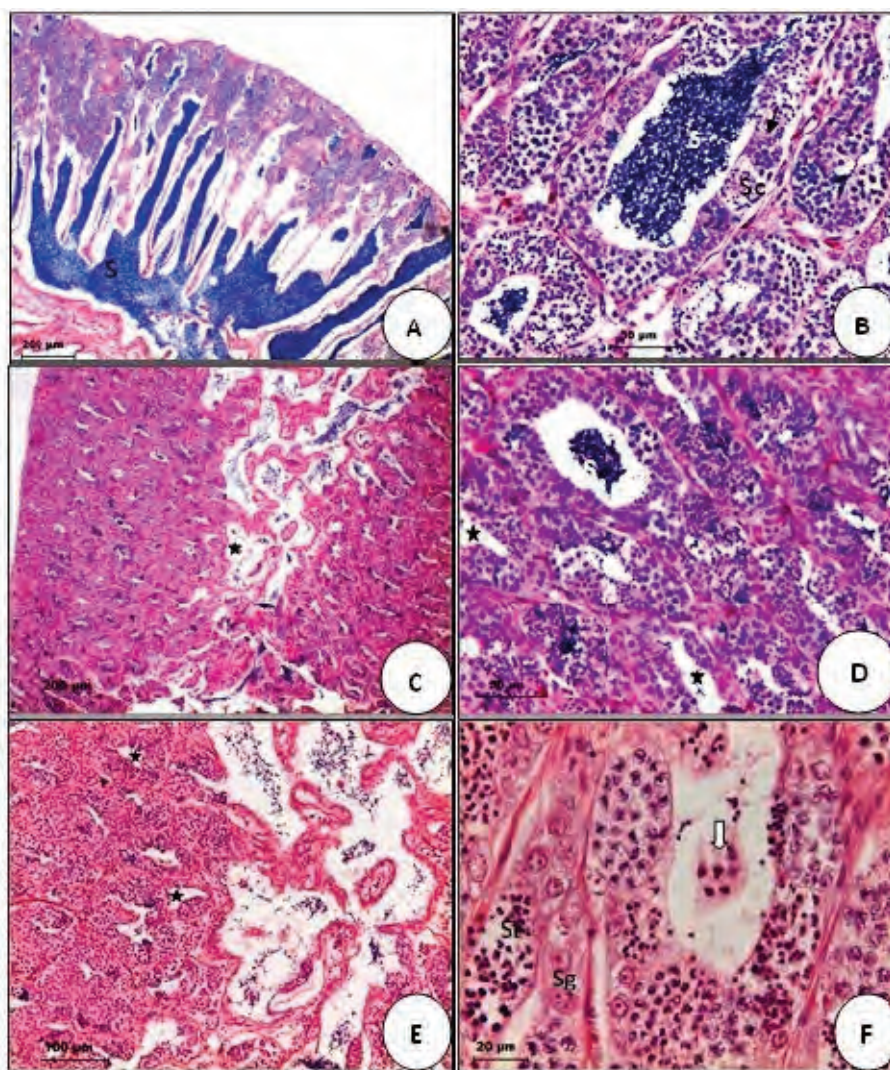


Fig. 1. Transverse sections of testis of adult male tilapia stained by H&E showing (A-B): control group; the seminiferous tubules filled with spermatozoa. (C-D): 0.03 mg/L⁻¹ dimethoate group showing decrease in sperm quantity with narrow tubular lumen (asterisk). At 5.00 mg/L⁻¹ exposed group (E-F) the seminiferous tubules appeared empty (asterisk) and degenerated cells debris were found in the lumen (white arrow). S: spermatozoa; SC: spermatogenic cyst.

tubules at Day 15 (Fig 1E). Moreover, specimens from Day 30, exposure fish displayed empty seminiferous tubules and degenerative cell debris were also observed in the lumen (Fig. 1F).

Pituitary morphology and LH immunohistochemistry

Pituitary morphology

In all studied tilapia, the adenohypophysis of the pituitary gland is divided into three regions: rostral pars distalis (RPD), proximal pars distalis (PPD), and the pars intermedia (PI). The neurohypophyseal nerve trunk invades the RPD and sends its complicated ramifications between the adenohypophyseal cells (Fig. 2A, C & E). In control group,

LH immunoreactive cells are distributed throughout the PPD, forming small clusters, cords and isolated cells surrounding the neurohypophyseal branches (Fig. 2A). The LH cells are large polygonal in shape, and often contain coarse secretory granules and an ovoid, eccentrically located nucleus (Fig 2B).

Pituitary LH cells in dimethoate exposed fish

Immunohistochemical examination of LH cells in dimethoate exposed *O. niloticus* revealed cytological variations includes reduction in cells size and mass distribution in adenohypophysis as well as vacuolation and degeneration in dose and time-dependent. At (0.03 mg/L⁻¹) exposed fish, the LH cells occupied a narrow zone in the PPD as well as

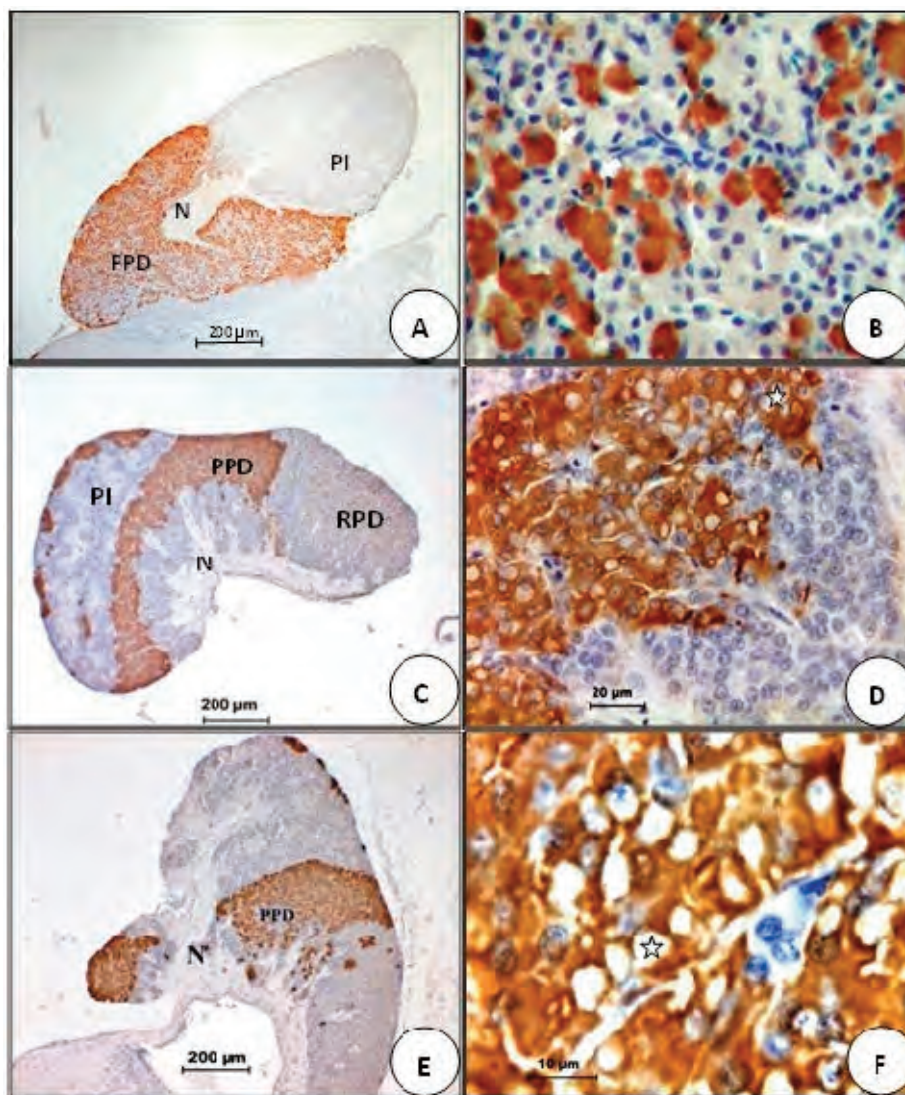


Fig. 2. Photomicrographs of the pituitary gland of *O. niloticus* immunostained with anti-human LH antiserum. (A): Control group showing large area of immunoreactive LH cells (B): The polygonal LH cells packed with dense granules (C-D): 0.03 mg/L⁻¹ dimethoate exposed fish showing strip of LH cells in middle of the gland and in PI (D): Some of LH cells appeared vacuolated (asterisk). At 5.00 mg/L⁻¹ dimethoate group (E-F) LH cells showed pronounced vacuolation (asterisk) the cells lost its membranes and appeared degenerated. PPD: proximal pars distalis; PI: pars intermedia; N: neurohypophysis; RPD: rostral pars distalis.

isolated cells in PI (Fig. 2C). The LH cells appeared hypotrophied with moderate vacuolation (Fig. 2C). After one month of exposure (5.00 mg/L⁻¹) specimens showed marked reduction in LH cells areas in the adenohypophysis, as well as weak immunoreactivity (Fig. 2E). The cells showed extensive degranulation in the cytoplasm forming large vacuoles and lost their membranes (Fig. 2F).

Discussion

The increase in human population necessitate the use of pesticides for enhancing crop production; which was reported to contribute about 50% of the total pollution source. In addition, the continuous release of sewage and industrial effluents exagger-

ates the problem of aquatic environment pollution (Pandey, 2000; Ngoula *et al.*, 2012). In this study, dimethoate acute toxicity to mature males Nile tilapia was assessed in order to evaluate the sub-lethal doses, which didn't cause fish death but affect their physiological, and reproductive functions (Auta and Ogueji, 2006; Abd El-Gawad *et al.*, 2011).

The hypophyseal-gonadal axis is the controlling system for reproduction of fishes as well as mammals through the pituitary gonadotropins, which are the critical components of the pituitary gonadal axis in all vertebrates that relay information from pituitary to the gonads (Karen *et al.*, 2011) regulating the timing and the process of reproduction. The mode of action of most the organophosphorus in-

secticides through inhibiting acetyl cholinesterase (Gore, 2001) and consequently; blocking transmission of nerve impulses. This effect could prevent the release of FSH and LH from the pituitary, resulting in reduced production of sperms (Short and Colborn, 1999; Gwynne, 2000). The results of pituitary hormones in this work, which showed a significant increase in the level of FSH and LH in the serum of the concentrations exposed males for 15 days, this may be due to that pituitary gland are evoked to compensate the normal levels of the hormone (Akhtar *et al.*, 1996). This was evident 30 days post exposure; where the level of FSH was almost similar to the value of the control. While LH levels in the serum showed continuous significant increase till the 30 days post exposure. This increase was associated with the degranulation and vacuolation of the LH cells in adenohypophysis.

Dimethoate induced a dose and time-dependent deleterious effects on the testes of tilapia, they decreased sperm amount in the seminiferous lumen at low dose (0.03 mg/L⁻¹), but emptied lumen at 5.00 mg/L⁻¹ 30 days post-exposure. Related reported effects regarding altered spermatogenesis caused by endosulfan include disruption of testicular lobules and damaged Sertoli cells in bluegill fish, *Lepomis macrochirus* (Dutta *et al.*, 2006), release of immature germ cells into the lobular lumen and abnormal preponderance of sperm and spermatogonia over intermediate germ cell stages in *Cichlasoma dimerus* (Da Cuna *et al.*, 2011, 2013). Presence of cell debris in the seminiferous tubule lumen constitutes signs of degenerative or necrotic processes. Tissue injury by the presence of endosulfan could lead to accumulation of cell debris including plasma membrane fragments, rich in cholesterol (Kisilevsky and Tam, 2003).

Organophosphorus pesticides including dimethoate disrupt the endocrine function by interfering with hormone synthesis, release, storage, transport and hormone receptor recognition/binding (Walker, 2003; Colborn, 2006). In the present study, the LH cells showed weak immunoreactivity, reduction of the areas along with vacuolation and degeneration in the dimethoate-exposed fish in a dose and time-dependent manner. Mousa and Mousa (1999) found a decline in numbers and size of LH cells in Nile tilapia in Lake Manzalah, a polluted hyperosmotic environment. Pituitary gonadotropin levels have been shown to alter due to exposure to organochlorine pesticides (Armenti *et*

al., 2008).

In several teleosts, gonadotropins not only regulate spermatogenesis via Sertoli cell function, but also influence the steroidogenic function of Leydig cells (Kamei *et al.*, 2005), possibly related in part; but not limited to the constitutive activity of LH receptors (Kwok *et al.*, 2005). Assuming a similar role of LH cells as a regulator for testes function in *O. niloticus*. Alterations in gonadotropins by dimethoate are more likely to lead to reproductive failure. The observed reproductive endocrine toxicity from dimethoate exposure might be due to pesticide interference with the pituitary-testicular axis.

Conclusion

Exposure of male Nile tilapia to sublethal or environmentally relevant concentrations of organophosphate dimethoate affects the structure of pituitary LH cells and consequently causes disturbances in testicular histophysiological features as evidenced from histological, hormonal and immunohistochemical investigations.

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